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(54) A method for purifying an outer membrane protein of Haemophilus influenza.

(57) A method of purifying an immunogenic outer membrane protein of H. flu. The steps comprising, formation of an insoluble fraction comprising the outer membrane protein associated with the peptidoglycan from the H. flu; digesting contaminated RNA from the insoluble fraction; separating the outer membrane protein from the insoluble fraction into the supernatant; and concentrating the solubilized outer membrane protein.

EP 0 389 925 A1

A METHOD FOR PURIFYING AN OUTER MEMBRANE PROTEIN OF HAEMOPHILUS INFLUENZAE

This application is a continuation-in-part of U.S. Serial No. 092,948 filed October 8, 1987 which is a continuation-in-part of U.S. Serial No. 932,872 filed November 18, 1986.

BACKGROUND OF THE INVENTION

Haemophilus influenzae type b has long been recognized as a frequent pathogen, particularly in infants and children, but only recently has nontypable H. influenzae been recognized as an important pathogen. It is now well established that nontypable H. influenzae causes pneumonia, bacteremia, meningitis, postpartum sepsis, and acute febrile tracheobronchitis in adults. In addition, nontypable H. influenzae causes neonatal sepsis and is a frequent etiologic agent in acute otitis media in infants and children. Therefore, the importance of discovering a method to assay a clinical sample such as sputum, cerebral spinal fluid, blood and others for the presence of H. influenzae is clear.

The observation that nontypable H. influenzae causes serious infections in adults and children has stimulated interest in study of the pathogenesis and potential virulence factors associated with this bacterium. The ribitol capsule of H. influenzae type b is a virulence factor for the organism, and antibody to capsule protects the host by means of bactericidal and/or opsonizing actions. These observations have generated much investigation on the role of the capsular polysaccharide in infection with H. influenzae type b and protection from these infections. However, nontypable H. influenzae lacks a polysaccharide capsule, and, similar to the outer membranes of other gram-negative bacteria, the outer membrane of H. influenzae is composed of outer membrane proteins (OMPs) and lipopolysaccharide (LPS). Therefore, studies of the relationship between virulence of nontypable H. influenzae and surface antigens focus on OMPs and LPS.

Analysis of OMPs of nontypable H. influenzae has shown that there are marked differences in OMP composition among strains. See e.g. Murphy et al, "A Subtyping System For Nontypable Haemophilus influenzae Based on Outer-membrane Proteins," J. Infect. Dis, 1983, 147:838-46; Barenkamp et al, "Outer Membrane Protein and Biotype Analysis of Pathogenic Nontypable Haemophilus influenzae," Infect. Immun, 1982, 36:535-40; Lorb et al, "Outer Membrane Protein Composition in Disease Isolates of Haemophilus influenzae, Pathogenic and Epidemiological Implications," Infect. Immun, 1980, 30:709-17.

A subtyping system for nontypable H. influenzae based on the major OMPs has previously been developed. A surface exposed antigen (immunogen) which is conserved in all strains is an important tool in developing a method of identifying H. influenzae in clinical specimens as well as a vaccine against H. influenzae.

A relatively simple method for purifying the conserved surface exposed antigen would be desirable. Methods that have been used included the step of initially isolating the outer membrane and the resultant products contained contaminating RNA, other cellular components and detergents. It is therefore an object of this invention to provide a method for purifying the conserved surface exposed antigen, which is relatively simple and produces an increased yield of the desired antigen. It is a further object of this invention to provide a purification method which will produce a product free of contaminating RNA and undesirable cellular components.

BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention there is provided a method for purifying an immunogenic outer membrane protein of H. influenzae (H. flu) comprising the steps of (1) obtaining a first fraction comprising the outer membrane protein associated with peptidoglycan directly from a suspension of H. flu in a suspending medium, said fraction being insoluble in said suspending medium; (2) separating said first fraction from the suspension; (3) digesting contaminating RNA from the first fraction by treating with a digesting material in a first liquid in which the peptidoglycan associated with the outer membrane protein is insoluble and in which digested RNA is soluble, to form a second fraction, said second fraction comprising the outer membrane protein and peptidoglycan; (4) separating said second fraction from said first liquid; (5) suspending the second fraction in a second liquid in which said outer membrane protein is soluble and the peptidoglycan is insoluble to obtain a third liquid comprising solubilized outer membrane protein; and (6)

separating the third liquid comprising the solubilized outer membrane protein from the insoluble peptidoglycan and concentrating the outer membrane protein.

DETAILED DESCRIPTION OF THE INVENTION

"Nontypable Haemophilus influenzae" as used herein, means H. influenzae which lacks a polysaccharide capsule and which has an outer membrane comprising outer membrane proteins (OMPs) and lipopolysaccharides (LPS).

"Immunogenic portion" means that portion which will result in an immunological antibody response in a host organism. Such portion may be considered an antigen.

"Epitope" means that limited immunogenic portion which results in a specific immunological response.

In accordance with the present invention, a mouse monoclonal antibody that recognizes an epitope on a 16,600-dalton outer membrane protein (p6) was developed to nontypable Haemophilus influenzae. This epitope was present on all 115 isolates of H. influenzae tested, including typable and nontypable strains. Screening of 89 strains of other bacteria demonstrated that this epitope is a highly specific marker for H. influenzae because the epitope was absent in virtually all other bacterial species tested. Western blot assays were performed with two normal human serum samples and convalescent-phase serum from an adult with bacteremia due to nontypable H. influenzae. Antibody to the 16,600-dalton outer membrane protein was present in all three human serum samples.

Prototype strains of nontypable H. influenzae representing the eight OMP subtypes were obtained from our own collection. See Murphy et al, supra. Strain 3524 was isolated from the sputum of a patient with chronic bronchitis at the Erie County Medical Center (Buffalo, NY). Dr. S. Berk (V.A. Medical Center, Mountain Home, Tenn) provided 14 strains of nontypable H. influenzae from blood or transtracheal aspirates. The remaining strains of nontypable H. influenzae were clinical isolates from the Erie County Medical Center and the Buffalo V.A. Medical Center.

Dr. J. Ward (University of California at Los Angeles) provided 54 strains of H. influenzae type b. The remaining strains of H. influenzae type b were clinical isolates from the Buffalo Children's Hospital. Reference strains of other capsular serotypes of H. influenzae were obtained from the Centers for Disease Control (Atlanta).

Copies of Haemophilus paraphrophilus ATCC 29240, Haemophilus segnis ATCC 10977, Haemophilus parainfluenzae ATCC 7901 and 9276, Haemophilus aegypticus ATCC 11116, Haemophilus parahemolyticus ATCC 10014, nontypable H. influenzae ATCC 19418, Actinobacillus actinomycetemcomitans ATCC 29522, ATCC 29523, ATCC 29524, NCTC 9707, and NCTC 9710, actinobacillus equili ATCC 19392, Actinobacillus seminis ATCC 15768, and Actinobacillus suis ATCC 15557 were provided by Dr. J. Zambon (School of Dentistry, State University of New York at Buffalo). Isolates of all other species were provided by the clinical microbiology laboratory at the Erie County Medical Center.

The identity of strains of H. influenzae was confirmed by colonial morphology and growth requirement for hemin and nicotinamide adenine dinucleotide. Capsular serotypes were determined by CIE with use of reference strains and antiserum from the Centers for Disease Control, Murphy et al., supra. Strains were stored in Mueller-Hinton broth plus 10% glycerol at -70° C.

BALB/c mice were immunized ip with 0.1 ml of 10^8 cells of nontypable H. influenzae strain 3524 on days 0 and 28. On day 32 after the initial immunization, selected animals were killed with chloroform, their spleens were removed, and splenic lymphocytes were harvested by perfusion of splenic pulp with minimal essential medium.

To achieve hybridoma development by fusion of the donar spleen cells to the NS 1 (nonsecreting variant of the IgG1 BA/c plasmacytome P3XAg8) plasmacytoma cells (obtained from the Salk Institute of Biology [La Jolla, Calif] under National Cancer Institute contract NO1-CB-23886), 35% polyethylene glycol was used in a modification of the procedure of Kennett, Cell Fusion, Methods Enzymol, 1979, 58:345-359. In brief, 10^7 spleen cells were combined with 10^6 NS-1 cells in minimal essential medium with serum. The cells were centrifuged at 170 g for 10 min at 25 C. All of the supernatant was removed, and the pellet was tapped to loosen it. Two-tenths milliliter of 35% polyethylene glycol 1,000 (Sigma Chemical Co., St. Louis) in minimal essential medium without serum was added and the mixture was stirred gently and left at 25C for 8 min, with the last 3 min consisting of centrifugation at 500 g to pellet the cells. At the end of the original 8 min, 5 ml of minimum essential medium (MEM) with serum was added and gently pipetted once to resuspend the pellet. The mixture was centrifuged at 250 g for 5 min at room temperature (25C). All of the supernatant was removed. Five milliliters of complete minimal essential medium (medium with glucose

[4.5 mg/ml] and 20% fetal bovine serum) was added to resuspend the pellet. The mixture was transferred to a 25-ml Erlenmy r flask containing the appropriate amount of compl te minimal essential medium to obtain 3 x 10^5 plasmacytoma cells/ml. The cells were stirred gently and distributed in 0.05-ml samples into microtiter wells.

At 24 hr after the polyethylene glycol fusion, 0.05 ml of medium containing hypoxanthine (13.6 µg/ml), aminopterin (0.36 µg/ml), and thymidine (3.87 µ/ml) was added to each well. The microtiter plates were placed in a tissue culture incubator at 85% humidity in an atmosphere of 5% CO_2 and 95% room air. Fresh medium containing hypoxanthine, aminopterin, and thymidine was added on day 7, and plates were checked for macroscopic plaques after day 10. The supernatant from all wells was tested for the presence of antibody with an ELISA (enzyme linked minimal absorbent assay).

ELISAs were performed in polyvinyl 96-well microtiter plates (Dynatech, Alexandria, VA); 200-µl volumes were used for each step. Wells were coated with a cell envelope preparation (10 µg/ml) of nontypable H. influenzae strain 3524 prepared by the method of Johnston, "immunobiology of Neisseriagonorrhocae", American Society for Microbiology, 1978, 121-9. Plates were incubated at 37C for 1 hr followed by overnight incubation at 4 C. Wells were washed three times with PBS (phosphate buffered saline) plus 0.05% Tween 20^R surfactant between each step. Unbound sites on the plastic were blocked with 3% bovine serum albumin in PBS for 2 hr at 37C. Tissue culture supernatants (or dilutions of mouse ascites fluid in subsequent experiments) containing monoclonal antibody were incubated in the wells overnight at 4C. Rabbit antibody to mouse IgG and IgM was then incubated for 2 hr at 37C followed by protein A-peroxidase for 2 hr at 37C. Two hundred microliters of substrate was then added to each well. Substrate was prepared by dissolving 10 mg of o-phenyl-enediamine in 1 ml of methanol and adding this solution to 99 ml of citrate-phosphate buffer, pH 5.0, plus 0.1 ml of 3% H_2O_2. After the substrate was incubated for 45 min in the dark at room temperature, the reaction was stopped with 50 µl of 4 N H_2SO_4. The OD_{490} was measured. Each set of ELISAs was performed with a control in which NS-1 tissue culture supernatant or ascites fluid was used in place of the monoclonal antibody being tested. On the basis of the results of ELISA screening, selected clones were propagated by subsequent transfer to larger tissue culture wells. Large quantities of antibody were produced in tissue culture and by ip injection of 10^5 hybridoma cells into pristane-primed BALB/c mice. The resulting ascitic fluid was harvested in three to four weeks and tested for specificity.

The strains to be assayed were grown on chocolate agar (or other appropriate medium, depending on the species) overnight at 37C in an atmosphere of 95% room air and 5% CO_2. Cells from one plate were harvested by suspension in PBS and centrifugation at 10,000 g for 20 min. The resulting pellet was suspended in enough PBS to allow the suspension to be drawn into a micropipette. One-tenth milliliter of the suspension of bacteria was added to 0.4 ml of sample buffer (0.06 M Tris, 1.2% SDS, 1% B-mercaptoethanol, and 11.9% glycerol) and heated for 5 min in a boiling water bath. The resulting organisms are referred to as whole cell preparation.

A 10-µl drop of whole cell preparation was placed on a nitrocellulose sheet (Schleicher and Schuell, Inc., Keene, NH) and allowed to air-dry. The sheet was then placed in 3% gelatin in Buffer A (0.012 M Tris and 0.15 M NaCl, pH 7.4) for 1 hr. After the sheet was rinsed with Buffer A, it was placed in an appropriate dilution of antibody and allowed to shake at room temperature overnight. The sheet was rinsed with Buffer A and placed in 1:3,000 dilution of protein A peroxidase (Zymed Laboratories, San Francisco) and shaken for 1 hr at room temperature. The sheet was rinsed and immersed in horseradish peroxidase color development solution (0.015% H_2O_2; Bio-Rad, Richmond, Calif) for 45 min. Controls assayed on each sheet included sample buffer (negative control). A negative result was recorded when the dot was no different from the background color, and a positive result was recorded when the dot turned purple-blue. About 90% of dot assays were unequivocally positive or negative. Those strains that yielded equivocal results in the dot assay were subjected to Western blot assay.

Preparation of LPS.

Lipopolysaccharide (LPS) was prepared from nontypable H. influenzae strain 3524 by two methods. The first method was a modification of the phenol-water extraction method of Westphal and Jann, "Bacterial Lipopolysaccharides", Methods in Carbohydrate Chemistry, 1965, 5:83-91. The second method was that of Hitchcock and Brown, Journal of Bacteriology, 1983, 154:269-77. The latter method uses the enzyme proteinase K (Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany), which hydrolyzes proteins but has no effect on LPS.

Whole cell and LPS preparations were subjected to SDS-PAGE (sodium dodecyl sulfate polyacrylamide

gel electrophoresis) with either 11% or 13.2% separating gels, Murphy et al, supra. When electrophoresis was completed, the gel was placed with a nitrocellulose sheet that had been previously boiled in distilled water, and the sheet was immersed in 0.3 M sodium citrate plus 3 M NaCl. Electrophoretic transfer was carried out in a Trans-Blot^{R} cell (Bio-Rad) at 50 V for 90 min. The electrode buffer was 0.025 M Tris, pH 8.3, 0.192 M glycine, and 20% methanol. The nitrocellulose sheet was then treated exactly as described for the dot assay; it was blocked with 3% gelatin and incubated sequentially with antibody 7F3, protein A-peroxidase, and substrate horseradish peroxidase color developer.

I radiolabeling of surface OMPs.

Extrinsic labeling of surface-exposed OMPs was accomplished with a lactoperoxidase-catalyzed radioiodination procedure, Hansen et al, Infect. Immun. 1981, 32:1084-92.

The ELISA with outer membranes of nontypable H. influenzae strain 3524 coated on microtiter plates demonstrated that the hybridoma designated 7F3 was producing antibody 7F3 that recognized a determinant in the outer membrane of the bacterium. Gel immunodiffusion indicated that this antibody was of the IgG3 isotype. Figure 1 shows a Western blot that indicates that the determinant recognized by antibody 7F3 was on a protein with a molecular size of 16,600 daltons; lane A shows molecular weight standards on the nitrocellulose sheet, and lane B shows the 16,600-dalton protein recognized by antibody 7F3 in a whole cell preparation of nontypable H. influenzae strain 3524. Specifically, lane A shows molecular weight standards transferred from a 13.2% gel; lane B shows a whole cell preparation of nontypable H. influenzae strain 3524 incubated with antibody 7F3, protein A-peroxidase, and peroxide substrate; and lane C is an autoradiograph of a whole cell preparation of nontypable H. influenzae strain 3524 made from bacteria extrinsically labeled with ^{125}I. All three lanes were from the same gel. Western blot assay done by this method in 25 strains of H. influenzae showed that antibody 7F3 recognized a determinant on this 16,600-dalton protein in every strain. Because the antibody recognized a determinant on a protein of identical molecular size in multiple strains, we screened larger numbers of strains with use of a dot assay rather than Western blot.

To determine whether the protein recognized by antibody 7F3 could be extrinsically labeled, we labeled nontypable H. influenzae strain 3524 with ^{125}I. The proteins were subjected to SDS-PAGE and transferred to a nitrocellulose sheet. One lane was exposed to x-ray film, and one lane was incubated with 7F3, protein A-peroxidase conjugate, and substrate. Figure 1 shows that the band recognized by antibody 7F3 (lane B) corresponds to an ^{125}I-labeled band (lane C).

To assess further whether the epitope recognized by antibody 7F3 was on a protein or on LPS, we performed two additional experiments. An ELISA was performed as described above in which some wells were coated with a cell envelope preparation of nontypable H. influenzae strain 3524 and other wells were coated with LPS prepared from nontypable H. influenzae strain 3524 by the phenol-water method Westphal et al, supra. Antibody 7F3 was reactive with a cell envelope preparation (OD, 0.375) that contained OMPs and LPS, Johnston et al, supra, but was nonreactive with LPS (OD, 0.062). This finding indicates that the epitope recognized by antibody 7F3 resides on an OMP.

Figure 2 is a Western blot assay depicting another experiment designed to assess whether the epitope recognized by antibody 7F3 is on a protein or LPS. The lanes marked A contain LPS prepared by proteinase K lysis of cells of strain 3524, Hitchcock et al, J. Bacteriol, 1983, 154:269-77, the lanes marked D contains LPS of strain 3524 prepared by the phenol-water method, Westphal et al, supra, and the lanes marked C contain a whole cell preparation of strain 3524. All samples were assayed on the same gel and transferred to the same nitrocellulose sheet. Figure 2, left, was incubated with antibody 7F3 (ascites fluid dilution, 1:500), and figure 2, right, was incubated with antibody 3D2 (ascites fluid dilution, 1:500), a monoclonal antibody that recognizes the lipid A portion of H. influenzae LPS. Antibody 7F3 does not bind to either of the LPS preparations and binds only to a band with a molecular weight of 16,600 in the whole cell preparation. This observation demonstrates that antibody 7F3 recognizes an epitope on a protein and not on LPS.

Specifically, Figure 2 shows a Western blot assay from a 13.2% gel: (left) incubation with antibody 7F3 and (right) incubation with antibody 3D2, which recognizes an epitope on the lipid A of H. influenzae. The lanes marked A contain LPS of nontypable H. influenzae strain 3524 prepared by lysis of cells with proteinase K, the lanes marked B contain phenol-water prepared LPS of strain 3524, and the lanes marked C contain a whole cell preparation of strain 3524. Molecular weight standards are noted on the right.

Studies were performed to determine the species specificity of the antigen recognized by antibody 7F3. Whole cell preparations of 115 isolates of H. influenzae were studied by either dot assay or Western blot assay. The strains included 73 type b, 37 nontypable, and 1 each of types a and c-f. All 115 strains of H.

influenzae contained the epitope recognized by antibody 7F3, a result indicating that this epitope is a common antigen among strains of H. influenzae.

Sixty isolat s of various bacterial species were studied to determine wheth r this epitope is present in bact ria other than H. influenzae. All 60 of these strains lacked the determinant recognized by antibody 7F3 (Table 1).

TABLE 1

SPECIFICITY OF ANTIBODY 7F3 FOR VARIOUS BACTERIAL SPECIES		
Bacterium	No. tested	No. positive
Gram-negative		
Escherichia coli	10	0
Actinobacillus species	10	0
Proteus species	7	0
Pseudomonas species	5	0
Klebsiella species	4	0
Serratia species	4	0
Enterobacter cloacae	1	0
Morganella morganii	1	0
Neisseria gonorrhoeae	6	0
Neisseria species	2	0
Gram-positive		
Staphylococcus aureus	5	0
Staphylococcus species	2	0
Viridans streptococci	1	0
Streptococcus faecalis	1	0
Diphtheroids	1	0
Total	60	0

Twenty-nine strains of Haemophilus species other than H. influenzae were studied. Twenty-five of these isolates lacked the 7F3 epitope (table 2). Two strains of H. parahemolyticus contained the determinant. In addition, one strain of H. paraphrophilus and one of H. aegypticus contained a 20,000 dalton protein that was recognized by antibody 7F3.

TABLE 2

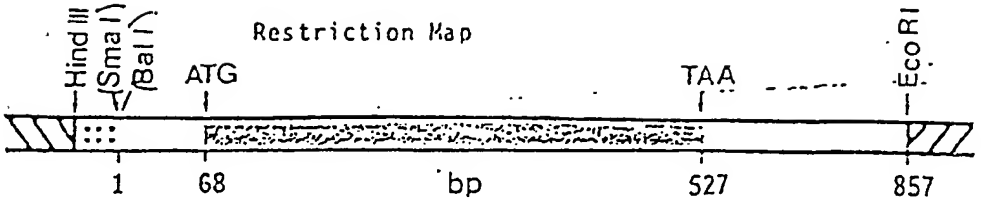
SPECIFICITY OF ANTIBODY 7F3 FOR VARIOUS SPECIES OF HAEMOPHILUS		
Species	No. tested	No. positive
H. parainfluenzae	24	0
H. parahemolyticus	2	2
H. paraphrophilus	1	1*
H. segnis	1	0
H. aegypticus	1	1*
Total	29	4

*In the Western blot assay, antibody 7F3 recognized a 20,000-dalton protein in these strains.

Human serum antibody.

Human serum was tested for the presence of antibody to the 16,600-dalton OMP by Western blot assay. Figure 3 shows whole cell preparations of nontypable H. influenzae strain 3524 that were assayed on the same gel and transferred to nitrocellulose paper; lane A was incubated with 7F3 ascites fluid and shows a single band corresponding to the 16,600-dalton protein, lanes B and C were incubated with two different samples of normal human serum, and lane D was incubated with serum obtained from an adult 17 days after bacteremia due to nontypable H. influenzae. All three samples of human serum have antibody to the 16,600-dalton OMP that contains the determinant recognized by antibody 7F3. The DNA sequence for the gene expressing this 16,600-dalton outer membrane protein is believed to begin at nucleotide 125 and continues until nucleotide 526. This amino acid sequence is included as a portion of the insert. The gene is believed to have the following sequence:

(a)



Amino Acid
DNA Sequence

(b)

```
CCCAAGTAAAATTTCCAGCTTGGTCTCCATACTTAACTAAATAAAAAACTCATTTAGGAGAAATCTA
1                                                                =====
met asn lys phe val lys ser leu leu val ala gly ser val ala ala leu
ATG AAC AAA TTT GTT AAA TCA TTA TTA GTT GCA GGT TCT GTA GCT GCA TTA
68                                                  100
ala ala cys ser ser ser asn asn asp ala ala gly asn gly ala gln gln
GCG GCT TGT AGT TCC TCT AAC AAC GAT GCT GCA GGC AAT GGT GCT CAA CAA
        125
thr phe gly gly tyr ser val ala asp leu gln gln arg tyr asn thr val
ACT TTT GGC GGA TAC TCT GTT GCT GAT CTT CAA CAA CGT TAC AAC ACC GTA
                                                    200
tyr phe gly phe asp lys tyr asp ile thr gly glu tyr val gln ile leu
TAT TTT GGT TTT GAT AAA TAC GAC ATC ACC GGT GAA TAC GTT CAA ATC TTA

asp ala his ala ala tyr leu asn ala thr pro ala ala lys val leu val
GAT GCG CAC GCA GCA TAT TTA AAT GCA ACG CCA GCT GCT AAA GTA TTA GTA
                                                300
glu gly asn thr asp glu arg gly thr pro glu tyr asn ile ala leu gly
GAA GGT AAT ACT GAT GAA CGT GGT ACA CCA GAA TAC AAC ATC GCA TTA GGA

gln arg arg ala asp ala val lys gly tyr leu ala gly lys gly val asp
CAA CGT CGT GCA GAT GCA GTT AAA GGT TAT TTA GCA GGT AAA GGT GTT GAT
                                                400
ala gly lys leu gly thr val ser tyr gly glu glu lys pro ala val leu
GCT GGT AAA TTA GGC ACA GTA TCT TAC GGT GAA GAA AAA CCT GCA GTA TTA

gly his asp glu ala ala tyr ser lys asn arg arg ala val leu ala tyr
GGT CAC GAT GAA GCT GCA TAT TCT AAA AAC CGT CGT GCA GTG TTA GCG TAC
                                                500
termination
TAA TTCTTGGTATTTCTAATACTTGAAAAACAGGATCCATTTTTTATTGGATCCTGTTTTGTTTTC

ATCGTTTGTAATTTAACCAATTAGCTTGAAAGAATGAATTTATTCTTTTGATTCTAAAATAAATGCG
          600

TTATCATTAACTCATCAACACAGTGGGTCGTTAGCTCAGTCGGTAGAGCAGCGGACTTTTAATCCGT
                                                    700

TGGTCGAAGGTTCGAATCCTTCACGACCCACCACTCTCTGATTTAATTGTCCAGTTGGGTTGTTAGCT

CAGTTGGTAGAGCAGCGGACTCTTAATTCGTCGGTCGAGAGTTCGAGCCTCTCACAACCTACCATTCT
          800

TACCG
```

It is worthy to note that this band is among the most prominent recognized by antibody in human serum.

Specifically, Figure 3 shows a Western blot assay from a 13.2% gel. All four lanes contain a whole cell preparation of nontypable H. influenzae strain 3524 from the same gel, but each lane was incubated with a different antiserum: lane A, antibody 7F3; lanes B and C, two different samples of normal human serum (dilution, 1:500); and lane D, serum obtained 17 days after bacteremia due to nontypable H. influenzae in an adult (dilution, 1:500). The incubation with antiserum was followed by incubation with protein A-peroxidase and peroxide substrate. The arrows indicate that all three samples of human serum contain antibody to the 16,600-dalton OMP that contains the 7F3 epitope. Molecular weight standards are indicated on the left.

In accordance with the invention, an IgG3 mouse monoclonal antibody that recognizes an epitope on a 16,600 dalton OMP on the surface of nontypable H. influenzae has been developed. This epitope is present in all 115 isolates of H. influenzae tested, including typable and nontypable strains. Screening of 60 strains of non-Haemophilus species demonstrated that the epitope is absent in all of these bacteria. The epitope was absent in 24 strains of H. parainfluenzae but was present in four to five strains of other Haemophilus species (table 2). These species are unusual pathogens in humans. Therefore, from the standpoint of clinically relevant isolates, antibody 7F3 is highly specific for H. influenzae.

This monoclonal antibody recognizing a common epitope that is highly specific for H. influenzae can be useful as a tool in the clinical microbiology laboratory. A rapid test to confirm the identity of a clinical isolate as H. influenzae (typable or nontypable) could be developed based on such an antibody. In order to construct a DNA probe to exploit this specific epitope, the DNA sequence of the gene encoding p6 is determined. Based on the DNA sequence, the amino acid sequence of the active p6 protein can be deduced. This information can be used to perform what is known as epitope mapping.

Epitope mapping involves the construction of a number of small peptides and testing these peptides for reactivity with monoclonal antibody 7F3. Since the epitope recognized by 7F3 is specific for H. influenzae, the corresponding peptide recognized by that antibody represents the specific determinant on H. influenzae. Once the amino acid sequence of the peptide is known, the DNA sequence of that segment can be deduced. Since H. influenzae contains the gene which codes for this epitope, the bacterium is known to contain DNA which has a sequence corresponding to this sequence. A DNA probe can, therefore, be constructed to correspond to the nucleic acids which code for the specific epitope on p6. Once the probe is constructed, it can be labeled, for example, with a radioactive member. This probe could then be used to assay a clinical sample such as sputum, cerebral spinal fluid, blood and others for the presence of H. influenzae. This will be possible because the DNA probe will bind to its complementary base pairs which are present in the genome of H. influenzae. Once this probe is constructed, this approach would represent an advantage over the current, widely used method of demonstrating growth requirements for hemin and nicotinamide adenine dinucleotide. An assay with a specific monoclonal antibody would yield results 24 hrs sooner.

OMPs and LPS are closely associated on the outer membranes of gram-negative bacteria. This fact and the observation that the determinant recognized by antibody 7F3 is in the molecular weight range where LPS separates lead one to question whether this determinant is on a protein or on LPS. Several lines of evidence indicate that the epitope recognized by antibody 7F3 is on a protein. First, staining with Coomassie blue of SDS gels demonstrated the presence of a band recognized by antibody 7F3 at 16,600 daltons in all strains of H. influenzae. Because Coomassie blue stains protein but not LPS, this observation is presumptive evidence that antibody 7F3 recognizes a protein determinant. Second, the configuration of the band on SDS-PAGE and Western blot was typical for protein; LPS showed multiple bands that were generally less distinct than the band at which the antibody 7F3 epitope resides. This point is further corroborated by the observation that monoclonal antibodies that recognize LPS determinants showed the typical "LPS" pattern in Western blot assays of whole cell preparations, in contrast to the well-defined single band recognized by antibody 7F3. Third, by ELISA, antibody 7F3 showed reactivity with cell envelope preparations that contain OMPs plus LPS, but the antibody showed no reactivity with isolated LPS. Finally, in the Western blot assay (figure 2), antibody 7F3 recognized a band in a whole cell preparation but failed to recognize determinants on LPS that was prepared by using two different methods. Taken together, these observations indicate that the epitope recognized by antibody 7F3 resides on an OMP.

To assess whether the OMP containing the antibody 7F3 epitope was surface exposed, OMPs were labeled by using a lactoperoxidase-catalyzed radioiodination procedure, Hansen et al, supra. Figure 1 shows that the protein containing the antibody 7F3 epitope is radiolabeled. This observation suggests that this 16,600-dalton OMP is surface exposed. For the purposes herein "surface exposed" or "outer membrane" means available for antibody binding.

The OMPs of nontypable H. influenzae show substantial strain-to-strain variability, as demonstrated by

SDS-PAGE analysis. This variability in the major OMPs in th 32,000-42,000-dalton range is th basis of the subtyping system for nontypable H. influenzae, Murphy et al, supra. It is of interest that studies of OMPs of H. influenza from three laboratories hav independently noted the presence of a "16,600"-dalton OMP in all strains of H. influenzae studied, Murphy et al, Barenkamp et al and Loeb et al, supra. It is this prot in that contains the antigenic determinant recognized by antibody 7F3. The present study indicates that the epitope recognized by antibody 7F3 on this low-molecular-weight OMP is an antigen common to all strains of H. influenzae. Identifying common surface antigens among strains is useful from the point of view of vaccine development because immunization with a single common antigen might induce protection from disease due to many strains. In addition, the observation that this 16,600-dalton protein has varied far less than other OMPs in the course of evolution leads to the speculation that this protein serves an important function for the bacterium and that its function is closely related to conservation of its structure.

The outer membranes of gram-negative bacteria are immunologically important structures because of their accessibility to host defense mechanisms. Indeed, antibody to OMPs of H. influenzae type b are widely prevalent in adults and are detected in the serum of infants who are convalescing from infections with H. influenzae. It has now been demonstrated that antibody to a 16,600-dalton OMP (p6) is present in human serum (figure 3). The presence of antibody to this OMP in normal human serum suggests that the OMP is important with regard to the human antibody response to H. influenzae.

Several observations suggest that p6 is an important target in immunity to Haemophilus influenzae:

1) Antibody raised from p6 isolated from a type b strain protects in an infant rat model.

2) A monoclonal antibody, 7F3, directed against p6 blocks human bactericidal activity against Haemophilus influenzae (NtHi).

3) Depleting normal human sera of p6 by affinity chromatography resulted in reduced bactericidal activity of that sera for Haemophilus influenzae.

4) And, immunopurified antibody to p6 from human sera was bactericidal.

p6 may be cloned molecularly using H. influenzae as a source of bacterial chromosomal DNA, lambda gtll bacteriophage as the vector in construction of the cenomic library, pUC18 plasmid as the vector used in subcloning the gene to facilitate sequencing, and E. coli as the host strains. The results allow further analysis of the molecular basis of both experimental and human immunity to p6 and permits large quantities of p6 to be produced once it is approved for use in vaccine against Haemophilus influenzae.

Molecular Cloning of p6:

The 16,600 dalton protein, designated herein as p6, is therefore present in the outer membranes of both typable and nontypable strains of Haemophilus influenzae and may be an important target in immunity to Haemophilus influenzae. The DNA sequence for the gene expressing this 16,600-dalton outer membrane protein is believed to begin at nucleotide 125 and continues until nucleotide 526. p6 is cloned molecularly using a nontypable strain of Haemophilus influenzae as a source of bacterial chromosomal DNA, lambda gtll bacteriophage as the vector in construction of the genomic library, pUC 18 plasmid as the vector in subcloning the gene to facilitate sequencing, and E. coli as the host strain. The monoclonal antibodies previously discussed and a polyclonal antiserum were used to screen for expression of p6. A portion of the genomic library was screened resulting in the detection of four positive recombinants. One, clone O, appears to produce a full length gene product expressed in high frequency. The DNA insert of this clone was used to subclone the gene into a plasmid vector. An E. coli transformant, 7-9B, also appears to express a full length gene product. It is likely that transcription is initiated from the actual promoter of the p6 gene, since both clone O and transformant 7-9B express the gene product in both the uninduced and induced states. Isolating and sequencing the gene for p6 allows for further analysis of the molecular basis of both experimental and human immunity to p6.

More specifically, recombinant DNA technology was used to clone the gene for the 16,600 dalton surface protein, p6, of nontypable Haemophilus influenzae (NtHi) into Escherichia coli. Chromosomal DNA from a clinical isolate was sheared, ligated to lambda gtll arms and packaged into phage heads. Four recombinant phages were detected by screening with monoclonal antibodies and a polyclonal antiserum. One, clone O, was restricted with $EcoR_1$ and ligated to plasmid vector pUC18 to facilitate sequencing. E. coli carrying recombinant plasmids were screened resulting in one positive, 7-9B. Both clone O and 7-9B produce a protein with an apparent molecular weight equal to or similar to native p6 as determined by Western blot analyses. In screening it was determined that transcription and translation of the Haemophilus influenzae p6 gene(s) were not dependent on the lac operator and promoter of either vector. Using immunofluorescence, the recombinant gene product's p6 epitopes could be localized on the surface of

these E. coli and accessible to antibody.

Haemophilus influenzae strain 1479 was grown at 37 degrees C in brain heart infusion broth supplemented with hem (10μg/ml) and nicotinamide adenine dinucleotide (10μg/ml).

The E. coli strain y1090 (r^-m^+) was used for the lytic growth of bacteriophage lambda gtll and strain JM83 as the host for the plamid pUC18. The E. coli strains were grown in L-broth (LB) or on LB agar with or without 50μg/ml of ampicillin, depending on the host strain. A more detailed description for the use of the respective host strains can be found elsewhere. Young et al, Science 222: 778-782; Mersing Rec. DNA Tech. Bull. 2:43-48.

A pellet of Haemophilus influenzae 1479 cells from a 750ml culture was resuspended in 110mls of 10mM HEPES buffer, pH 7.4. To this mixture was added EDTA to a concentration of 5mM and SDS to a concentration of 0.5% w/v and then incubated at 60 degrees C for 30 minutes. This lysate was then digested with 0.5ml of pronase (10mg/ml) at 37 degrees C for 2 hours and then subjected to two phenol/CIAA extractions followed by one CIAA extraction. Sodium chloride was added to a concentration of 0.2M to the aqueous phase, and DNA was precipitated with 2.5 volumes of chilled ethanol. Following precipitation in the cold, the DNA was pelleted by centrifugation, resuspended in Tris-EDTA buffer, and treated with DNase-free RNase at a concentration of 0.1 mg/ml at 37 degrees C for 1 hour. Finally, the DNA was extracted with phenol/CIAA, precipitated with sodium chloride and ethanol, and pelleted by centrifugation. The DNA was resuspended in Tris-EDTA buffer, measured for concentration by A_{260}/A_{280} and stored at 4 degrees C.

The phage library was screened with monoclonal antibody 7F3. Also used in screening was rabbit polyclonal antiserum produced by immunizations with solubilized p6 preparations of Haemophilus influenzae strain 1808.

Construction of the Haemophilus influenza 1479 genomic library:

The strategies for the construction of the library, depicted in Figure 4, were essentially those described by Young and Davis, 1985, Vol. 7, pp 29-41, Genetic Engineering, Plenum Press, N.Y. Haemophilus influenzae 1479 DNA was sheared by sonication with one 10 second burst (output control setting at 2) to an average length of 2-4 kilobase pairs (kb). The degree of shear was monitored by agarose gel electrophoresis. The Eco R1 sites of 50μg of this sheared DNA were methylated using Eco R methylase. The ends of the methylated DNA were made flush by the addition of Klenow polymerase and deoxynucleotide triphosphates. Following this reaction and the addition of sodium acetate to a concentration of 0.3M, the DNA was precipitated. After centrifugation, the pellet was resuspended in Tris-EDTA buffer. The DNA was then ligated to Eco R1 linkers (Bethesda Research Laboratories, Bethesda, Maryland) that had been phosphorylated. The ligation reaction was terminated by heating the mixture to 70 degrees C and then the excess Eco R1 linkers were digested using an excess of Eco R1. The methylated Haemophilus influenza 1479 DNA blunt end-ligated to Eco R1 linkers was purified from excess linkers by passage over a gel filtration column (Biogel p60, BIO RAD laboratories, Richmond, CA) using a column buffer containing 10mM Tris pH 7.5, 100mM NaCl, 1mM EDTA. Fractions were monitored by A_{280} and agarose gel electrophoresis. Fractions containing DNA of desired size range were pooled, and precipitated. The DNA was pelleted by centrifugation and resuspended in 4μl of Tris-EDTA buffer. The DNA was ligated to 3μg of dephosphorylated lambda gtll arms (STRATAGENE Cloning Systems, San Diego, CA) in a total reaction volume of 10μl. The ligation mixture was packaged using two packaging extracts according to the directions of the manufacturer (Gigapack™, STRATAGENE Cloning Systems). Packaged phage were plated on E. coli strain y1090 to determine the titer of plaque forming units and to determine the non-recombinant background by growth with IPTG and X-gal on LB + AMP plates. The library contains approximately $1.5X10^6$ independent recombinant clones with a background of less than or equal to 5%.

Screening the library:

Figure 5 depicts the methods used in screening. A portion of the y1090 plating stock, 0.2ml of a y1090 pellet resuspended in 10mM $MgSO_4$, was infected with $1.5X10^3$ pfus of the lambda gtll library for each 85mm plate. d Following the adsorption incubation, the cells were mixed with LB-agarose buffer, poured and spread evenly onto an LB + AMP plate. The plates were then incubated at 42 degrees C for 3 hours. Each plate was then overlaid with a dry nitrocellulose filter disk which had been saturated previously in 10mM IPTG. The plates were then incubated for 3 hours at 37 degrees C. Before removing the filters, the

orientation was marked and the filters and respective plates were labeled. The filters were rinsed briefly with Buffer A (0.01M Tris, 0.15M NaCl, pH 7.4) and placed in 3% gelatin in Buffer A for 1 hour. After the filters were rinsed again with Buffer A, they were incubated in a screening mixture of antibodies overnight at room temperature. The screening mixture was Buffer A containing 7F3 ascites fluid, at titers of 1:1000. The monoclonal antibody used shared no crossreactivity with the E. coli host strains while anti-1808 antiserum required a working dilution of 1:10,000 to maintain sensitivity and specificity. The filters were rinsed with Buffer A and placed in a 1:3000 dilution of protein A-peroxidase conjugate and shaken for 1 hour at room temperature. The filters were again rinsed with Buffer A, then immersed in horseradish peroxidase color development solution (0.15% H_2O_2; BIO RAD, Richmond, CA) for 45 minutes. plaques that appeared positive were removed from their respective plates, resuspended in 500µls of SM buffer, and rescreened. Plaques that were positive in the rescreening were then rescreened again but against the individual antibodies rather than the screening mixture.

Western blot analysis:

The Haemophilus influenza control, y1090 recombinants, and the molecular weight standards were prepared by heating at 100 degrees C for 5 minutes in a sample buffer containing 0.06M Tris pH 6.8, 1.2% SDS, 5% beta-mercaptoethanol, 11.9% glycerol and 0.003% bromophenol blue. The preparations were subjected to SDS-PAGE on a 15% separating gel. Gels were placed on a nitrocellulose sheet which had been previously boiled in distilled water and immersed in a 0.3M sodium citrate, 3M NaCl solution. Electrophoretic transfer was done using a Transphor^R electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA) at 50 volts for 90 minutes, in a buffer of 0.025M Tris, pH 8.3, 0.192M glycine and 20% methanol. The blocking, with subsequent additions of antibody, conjugate, and substrate development was performed in the same manner as described in the plaque screening.

Subcloning into a plasmid vector:

A strategy was devised to facilitate sequencing. The DNA insert (which consists of 867 residues), of a recombinant phage expressing p6 epitopes (as determined by screening), was subcloned into a plasmid vector. pUC18 was chosen as the plasmid vector for subcloning for several reasons including a means of selection, an inducible promotor, and an Eco R1 restriction site, features shared by phage cloning vector lambda gtll. The DNA of a recombinant phage was restricted with Eco R1 and then ligated to pUC18 which had been restricted with Eco R1 and dephosphorylated using calf intestinal alkaline phosphatase. The ligation mixture was used to transform competent E. coli strain JM 83. Transformants were selected for by growth on LB + AMP plates overlaid with IPTG and X-gal. White colonies, thought to represent JM 83 containing a plasmid plus insert, were individually picked and transferred to wells of microtiter plates containing L-broth + AMP + 10% glycerol. The plates were incubated overnight at 37 degrees C. A comb device was used to innoculate from the microtiter plates onto nitrocellulose sheets, previously immersed in IPTG, overlaying LB + AMP plates. The plates were incubated overnight at 37 degrees C, and then the nitrocellulose sheets were removed. The nitrocellulose was hung for 15 minutes in a chamber containing chloroform vapors to lyse the colonies, blocked in 3% gelatin containing 40µg/ml lysozyme and screened in the same way as the genomic library.

Results:

Screening the genomic library and characterization of recombinants.

Approximately 45,000 plaques were screened, the remainder of the unamplified library being frozen in aliquots at -70 degrees C in 7% DMSO. Four reactive clones designated as lambda gtll-Haemophilus influenzae 1479 clones O, P, 8, and 10, were found. Efforts focused on clones O and P since they appear to express gene products that are in larger quantities than 8 or 10 and/or more closely resemble the conformation of the native protein, p6.

Plates containing clones O and P were carefully scraped to harvest protein for Western blot analysis. Western blots show that both clones O and P produce a protein that is the same or similar in size to native

p6. However, clone O produces the protein in larger quantiti s when compared to clone P. Therefore, clone O was selected as the recombinant phage for the source of DNA to subclone into a plasmid vector.

Subcloninc into a plasmid vector and characterization of the transformant.

One thousand transformants, from the same transformation, were screened before one, 7-9B, was found to be positive. To make certain that 7-9B was in pure culture, positive colonies were picked, passaged and rescreened. Western blots of 7-9B grown on plates and in broth show that this recombinant also produces a gene product that is of an apparent molecular weight equal or similar to native p6. The recombinant plasmid Isolated from 7-9B, was restricted using Eco R1 and subject to agarose gel electrophoresis to determine the insert size. Restriction analysis, Figure 7, reveals the presence of a 2.5 kb DNA insert containing the p6 gene.

Using molecular cloning techniques, both recombinant phages and recombinant plasmid containing the gene encoding for Haemophilus influenzae 1479 p6, a 16.6K dalton outer membrane protein, was produced. The protein can be described by the nucleic acid sequence between nucleic acid 125 and nucleic acid 526, inclusively. Expression of the gene product by phage clone 0 and by recombinant plasmid 7-9B is independent of induction of the B-galactasidase promoter. This finding, coupled with Western blot analyses revealing apparently no difference in electrophoretic mobilities between the gene products of clone O and recombinant 7-9B, provide evidence for the likelihood that the recombinant gene product is initiated from its own, the p6 gene's, constitutive promoter.

The epitopes of the 16.6K dalton protein are accessible to monoclonal antibody 7F3 on the surface of E. coli transformant 7-9B.

Except as otherwise indicated all microbiological strains are generally available. All such strains as described herein are available from Dr. Timothy Murphy, Division of Infectious Diseases, State University of New York Clinical Center, 462 Grider Street, Buffalo, NY 14215. H. influenzae strains 3524 and 1479; E. coli strain JM83 containing plasmid 7-9B and hybridoma 7F3 are being deposited with the American Type Culture Collection (ATCC) at 12301 Parklawn Drive, Rockville, Maryland 20852 in accordance with the Budapest Treaty.

Purification of the Outer Membrane Protein of H. flu

A method for purifying the outer membrane protein of Haemophilus influenzae is disclosed in this invention.

The method disclosed herein is relatively simpler than previous methods. The method utilizes the whole bacterium as a starting material for incubation in detergent containing buffers, therefore eliminating the initial step of isolating the outer membrane, as was necessary in previous methods. By whole bacterium Is meant that the bacterium is used in an unprocessed state.

H. flu, in a suspending medium, is used to directly obtain a first fraction comprising the outer membrane protein associated with peptidoglycan. The suspending medium is a liquid in which the fraction is insoluble and may be a detergent buffer. By detergent buffer is meant a buffer solution comprising a detergent wherein the outer membrane protein and the peptidoglycan are insoluble. Suitable detergents include sodium dodecyl sulfate (SDS). An example of a suitable detergent buffer is Buffer B. Buffer B comprises 1% SDS, 0.1% Beta-mercaptoethanol, 0.01M tris, 0.5M NaCl, pH 8.0. The first fraction comprising the outer membrane protein (p6) associated with peptidoglycan is obtained by successively heat treating and centrifuging the suspension. The suspension may be sonicated briefly to help suspend the cells. Heat treatment may be by any suitable means known to those skilled in the art. An example of a suitable means for heat treatment is incubation at between about 20° C to about 37° C for between about 20 to 40 minutes. Centrifugation is at about 20,000g to about 40,000g for about 30-60 minutes at ambient temperature. Ambient temperature as used herein means between about 20° C to about 27° C. By successive heat treatment and centrifugation is meant that the H. flu, in the suspending medium, is heat treated then centrifuged then resuspended in the suspending medium and heat treated and centrifuged until the first fraction comprising the p6 associated with peptidoglycan is formed.

Contaminating RNA is then digested from the first fraction by use of a digesting material in a first liquid in which the peptidoglycan associated with the outer membrane protein remains insoluble and the digested contaminating RNA is soluble, such that a second fraction is formed. The second fraction is insoluble in the first liquid and comprises the outer membrane protein associated with p ptidoglycan. By contaminating

RNA is meant RNA which is present in the second fraction along with p6 and peptidoglycan. A digesting material allows for the digestion of contaminating RNA while the p6 remains in an insoluble form by virtue of its association with peptidoglycan. An example of a digesting material is ribonuclease A. The first liquid may be a detergent buffer as discussed supra. By soluble is meant selectively suspendable or removable with respect to the second fraction. Digesting material is added to the detergent buffer and the mixture is sequentially heat treated and centrifuged. Heat treatment and centrifuging are as described supra. By sequential heat treatment and centrifuging is meant that the first fraction is suspended in the detergent buffer comprising the digesting material and heat treated and centrifuged until contaminating RNA is digested and the second fraction is formed.

The outer membrane protein (p6) is separated from the second fraction by suspending in a second liquid in which said outer membrane protein is soluble and the peptidoglycan is insoluble and heat treating to release the outer membrane protein from the peptidoglycan to obtain a third liquid comprising the solubilized outer membrane protein. An example of a liquid in which said outer membrane protein is soluble and the peptidoglycan is insoluble is a detergent free buffer. By detergent free buffer is meant a buffer solution not having a detergent added. Examples of suitable detergent free buffer solutions are tris buffer, PBS and sodium tetraborate buffer. A preferred detergent free buffer is sodium tetraborate buffer. The second fraction is suspended in the detergent free buffer and heat treated at between about 50-75°C for about 30-60 minutes. The suspension is then centrifuged at about 80,000g to about 120,000g for a sufficient time at a sufficient temperature until a supernatant (third liquid) comprising the outer membrane protein is obtained. The supernatant is then recovered which comprises a soluble preparation of p6 which is free of undesirable cellular components. Undesirable cellular components include lipooligosaccharides, proteins other than p6, peptidoglycan, DNA and RNA.

The supernatant is then concentrated by means known to those skilled in the art and includes the method of pressure filtration.

The following example describes the manner and process of making and using the invention and set forth the best mode contemplated by the inventor of carrying out the invention, but is not to be construed as limiting.

Bacteria were grown in broth and 25 grams of bacteria were suspended in Buffer B, sonicated briefly to help suspend the cells and incubated at 37°C for 30 minutes. After incubation the suspension was centrifuged at 20,000g for 30 minutes at 25°C. The supernatant was discarded and the insoluble fraction was resuspended in Buffer B, incubated, and centrifuged as before. This cycle of incubations and centrifugations was repeated a total of 5 times. For the last two incubations, ribonuclease A (10 micrograms/ml) is added to Buffer B. Including the ribonuclease at this step allows for digestion of contaminating RNA while the p6 remains in an insoluble form by virtue of its association with peptidoglycan. In this way, the ribonuclease and the RNA degradation products remain soluble while p6 and peptidoglycan remain insoluble.

The p6/peptidoglycan insoluble fraction was suspended in 0.1M Sodium tetraborate buffer (pH 9.5) and incubated for 30 minutes at 65° C. The mixture was then centrifuged at 100,000 g for one hour at 35° C. This step releases p6 from peptidoglycan so that solubilized, p6 remains in the supernatant. This supernatant was then concentrated by pressure filtration approximately ten fold. The volume was brought back up to its original volume and reconcentrated. This concentrating and washing procedure was done five times using the borate buffer. The resulting p6 preparations have been characterized as follows: SDS-PAGE yields a single protein band at a molecular weight of approximately 16,000 daltons. The preparation is free of detectable lipooligosaccharide using a monoclonal antibody. An absorbance spectrum of the soluble p6 revealed a peak at a wavelength of 275 nM. Agarose-gel electrophoresis revealed that the preparation is free of DNA and RNA. Therefore, the final preparation contains purified, solubilized p6 protein from Haemophilus influenzae. Amino acid analysis of this material shows that the amino acid content is consistent with that predicted from the DNA sequence disclosed herein.

Other embodiments of the invention will be apparent to the skilled in the art from a consideration of this specification or practice of the invention disclosed herein. It should be understood that there may be other embodiments which fall within the spirit and scope of the invention as defined by the following claims.

Claims

1. A method for purifying an immunogenic outer membrane protein of H. flu comprising the steps of:

a) obtaining a first fraction comprising the outer membrane protein associated with peptidoglycan directly from a suspension of H. flu in a suspending medium, said fraction being insoluble in said

suspending medium;

b) separating the first fraction from the suspension;

c) digesting contaminating RNA from the first fraction by treating with a digesting material in a first liquid in which the peptidoglycan associated with the outer membrane protein is insoluble and in which digested RNA is soluble, to form a second fraction, said second fraction comprising the outer membrane protein and peptidoglycan;

d) separating said second fraction from said first liquid;

e) suspending the second fraction in a second liquid in which said outer membrane protein is soluble and the peptidoglycan is insoluble to obtain a third liquid comprising solubilized outer membrane protein; and

f) separating the third liquid comprising the solubilized outer membrane protein from the insoluble peptidoglycan and concentrating the outer membrane protein.

2. A method for purifying an immunogenic outer membrane protein of H. flu comprising the steps of:

a) harvesting the H. flu by suspending in a detergent buffer and successively heat treating and centrifuging to form an insoluble fraction comprising the outer membrane protein associated with peptidoglycan;

b) digesting contaminating RNA from the insoluble fraction formed in step a by treating with a digesting material in a detergent buffer in which the H. flu peptidoglycan associated with the outer membrane protein is insoluble and sequentially heat treating and centrifuging;

c) separating the outer membrane protein from the insoluble fraction formed in step b by suspending in a detergent free buffer, heat treating and centrifuging to obtain a supernatant comprising the outer membrane protein; and

d) concentrating the outer membrane protein in the supernatant.

3. The method as recited in Claim 2 wherein the outer membrane protein is p6.

4. The method as recited in Claim 3 wherein the insoluble fraction forming step comprises the steps of:

a) suspending H. flu cells in detergent buffer;

b) incubating at between about 20-37 ° C for about 20-40 minutes;

c) centrifuging at about 20,000 g to about 40,000 g for about 30-60 minutes at ambient temperature;

d) resuspending in the detergent buffer; and

e) repeating steps b and c for a sufficient number of times to form the insoluble fraction.

5. The method as recited in Claim 4 wherein the detergent buffer is Buffer B.

6. The method as recited in Claim 5 wherein Buffer B comprises 1 % SDS, 0.01 M tris, 0.5 M NaCl, 0.1 % β-mercaptoethanol, pH 8.0.

7. The method as recited in Claim 4 or 6 wherein the suspension step further comprises the step of sonicating for a sufficient time to aid in suspending of cells.

8. The method as recited in Claim 5 or 7 wherein the incubation is at 37 ° C for 30 minutes.

9. The method as recited in Claim 4, 7 or 8 wherein the centrifugation is at 20,000 g for 30 minutes.

10. The method as recited in Claim 3 or 9 wherein the digesting step comprises the steps of:

a) suspending the insoluble fraction formed in step 1 in the detergent buffer comprising a digesting material;

b) incubating at between about 20 - 37 ° C for about 20 -40 minutes;

c) contrifuging at about 20,000 g to about 40,000 g for about 30 - 60 minutes at ambient temperature;

d) discarding supernatant; and

e) repeating steps a, b, c, and d.

11. The method as recited in Claim 10 wherein the digesting material is ribonuclease A.

12. The method as recited in Claim 11, wherein the ribonuclease A is present in an amount of about 10 µg/ml of Buffer B.

13. The method as recited in Claim 10 or 11 wherein the incubation is at about 37 ° C for about 30 minutes.

14. The method as recited in Claim 10 or 11 wherein the centrifugation is at 20,000 g for 20 minutes.

15. The method as recited in Claim 3, 10 or 14 wherein the separating step comprises the steps of:

a) suspending the insoluble fraction in a detergent free buffer;

b) incubating at between about 50 - 75 ° C for about 30 -60 minutes;

c) centrifuging at about 80,000 to about 120,000 g for a sufficient time and at a sufficient temperature until a supernatant comprising the p6 is obtained; and

d) recovering the supernatant.

16. The method as recited in Claim 15 wherein the detergent free buffer is a 0.1 M sodium tetraborate solution having a pH of 9.5.

17. The method as recited in Claim 15 or 16 wherein the incubation is at 65 °C for 30 minutes.

18. The method as recited in Claim 15 or 17 wherein the centrifugation is at 100,000 g for 1 hour at 37 °C.

19. The method as recited in Claim 3 or 18 wherein the supernatant is concentrated by pressure filtration.

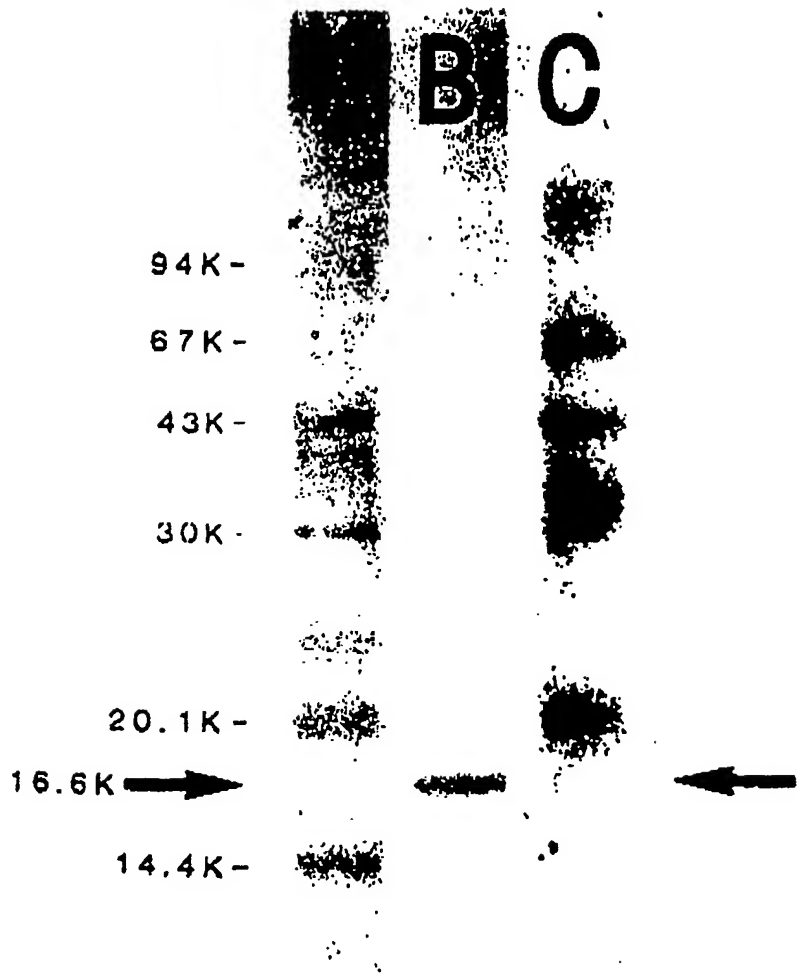


FIG. I

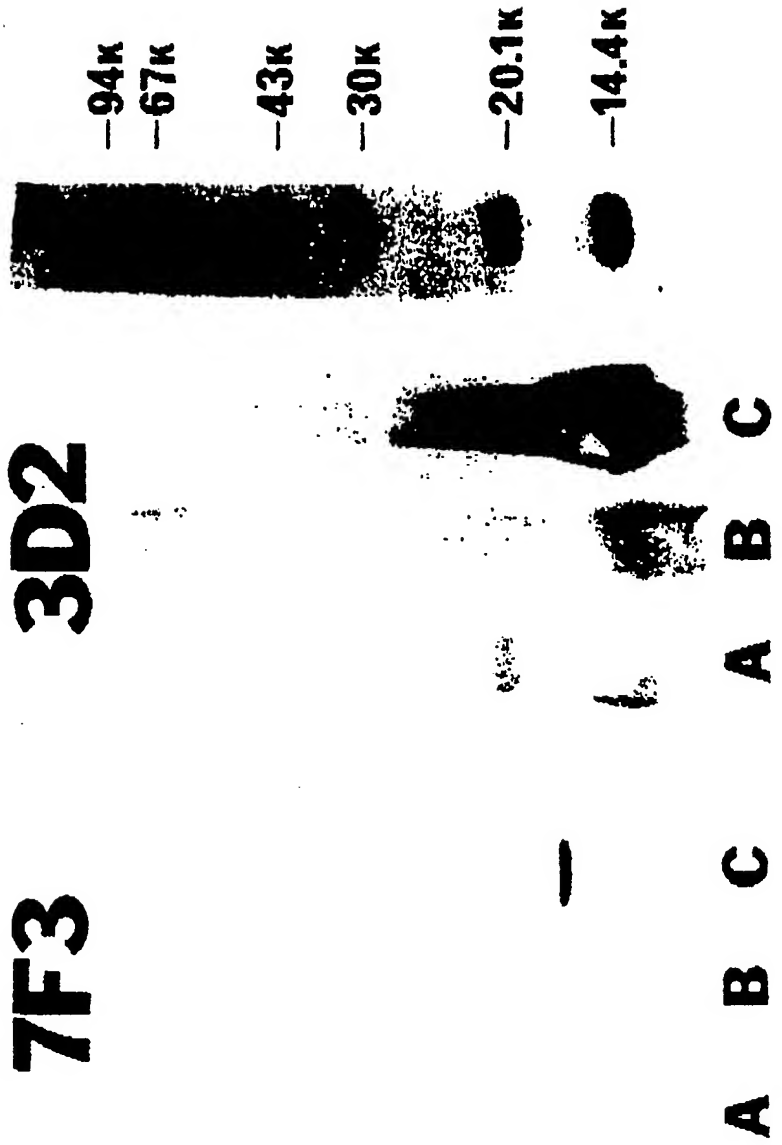


FIG.2

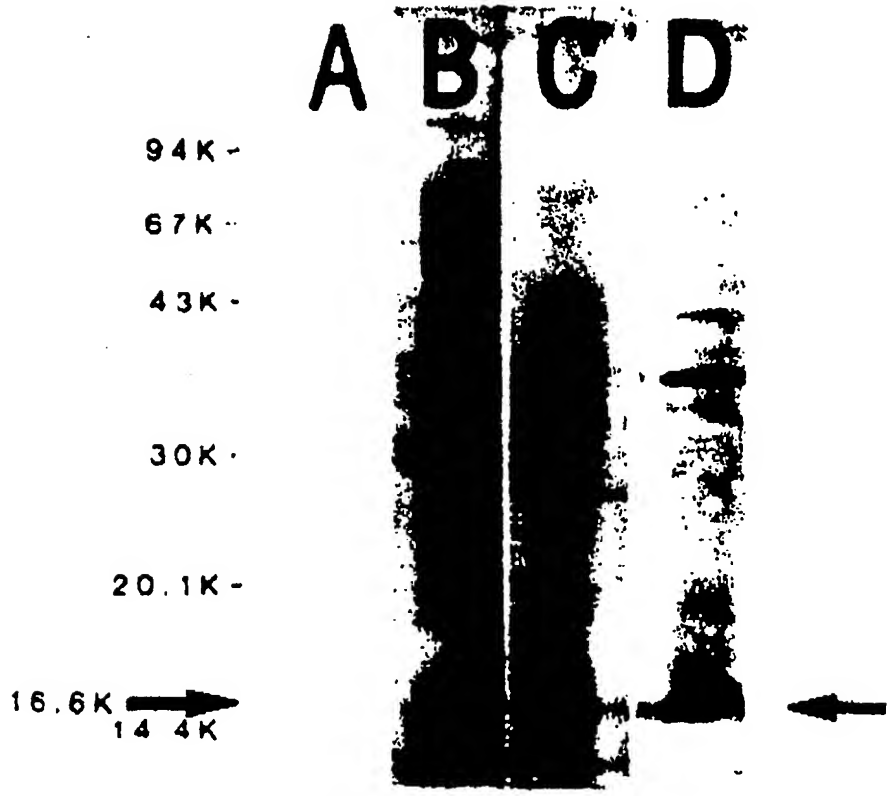


FIG.3

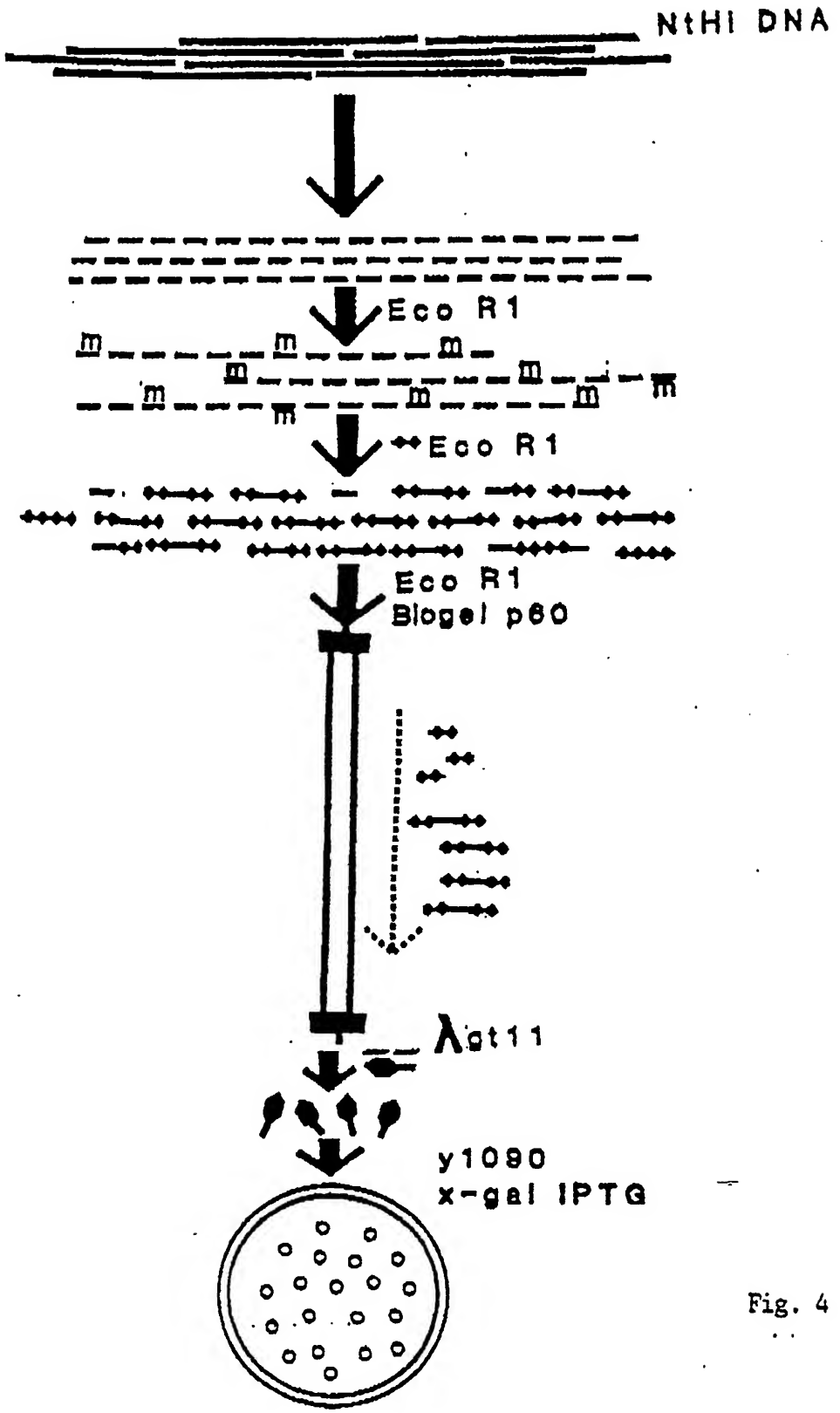


Fig. 4

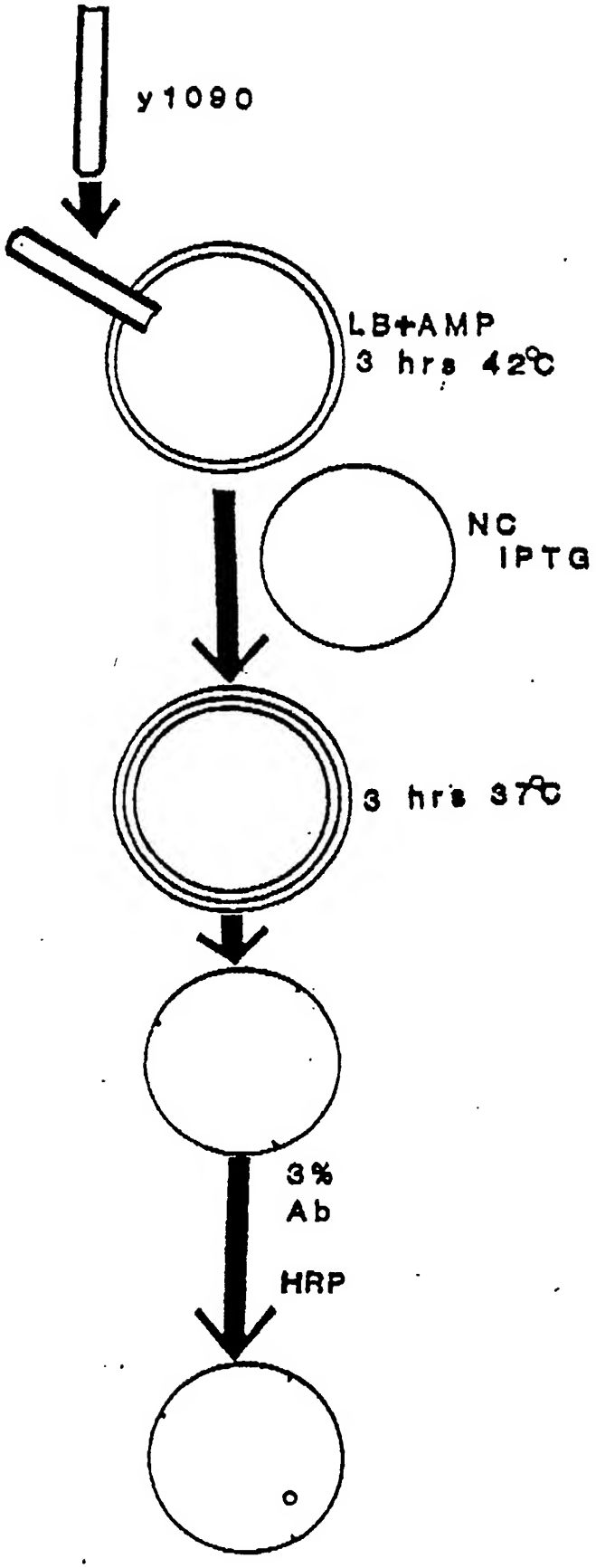


Fig. 5

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 90 10 5205

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Y	WO-A-8 804 932 (PRAXIS BIOLOGICS) * Page 29, line 30 - page 31, line 27; page 39, line 30 - page 42, line 19 * ---	1-19	C 07 K 3/12
Y	INFECTION AND IMMUNITY, vol. 54, no. 3, December 1986, pages 774-779, American Society for Microbiology; T.F. MURPHY et al.: "Antigenic characterization of the P6 protein of nontypable Haemophilus influenzae" * Page 774, right-hand columm, paragraph: "Materials and methods" - page 775, left-hand column, last line * -----	1-19	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	04-07-1990	REMPP G.L.E.

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